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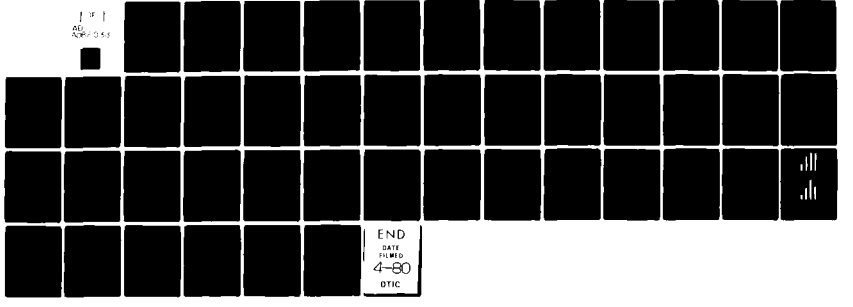

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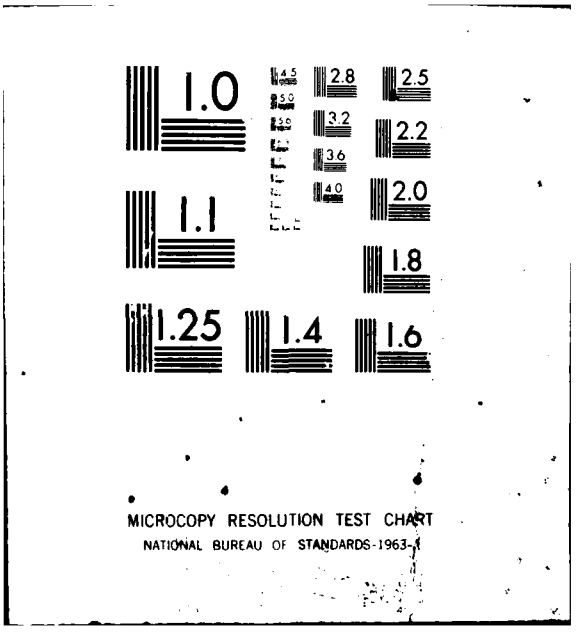
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KINETICS OF NEUTROPHIL-RELEASING ACTIVITY OF POST-LEUKOPHERESIS PLASMA

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We have shown in previous studies under this contract that granulocytopenia induced by removal of large numbers of granulocytes by filtration leukopheresis results in the triggering of granulocytopoietic mechanisms. These apparently normal homeostatic responses apparently operate at the end stage of granulopoiesis, i.e., mobilization of mature granulocytes in the circulating pool from the peripheral and probably bone marrow compartments. Subsequent studies

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20. Abstract

suggested the clinical use of autologous plasma from granulocyte donors to improve granulocyte yields in subsequent donations. The present studies have focused on assessing factors in the animal model developed by us which might bear an interpretation of results, not only in the animal, but ultimately in the human donor. These include the effect of deep vs light anesthesia, anticoagulation, and interaction of the blood with plastic cannulae. The maintenance of deep anesthesia is necessary if one wishes to eliminate a triggering of granulocyte mobilization, probably via the pituitary/adrenal axis. ~~We have~~ shown also that blood/plastic interaction also contributes to granulocyte mobilization, and this must be considered in interpreting some of these experiments. ~~We have~~ demonstrated that homologous plasma does not significantly affect the granulocyte count while heterologous plasma does, perhaps as a result of foreign protein interactions. The gluococorticoid, prednisolone, was shown to mobilize granulocytes in the rat to a degree similar to that in humans but at a much more rapid rate. ~~We have constructed~~ a mathematical model which gives a much more accurate measure of the magnitude of granulocyte mobilization. This model will be invaluable not only to better understand the results obtained in these experiments but will also be useful for the study of granulocyte kinetics in general. We have shown that the neutrophil releasing factor is probably not the "luekogenenol" of Dr. Frederick Rice. We are in the process of studying fractions of normal rat plasma prepared by Dr. Harvey Bank to determine which, if any, fraction of normal plasma has granulocyte mobilizing properties.

CONTENTS

INTRODUCTION

A primary goal of this contract is to determine unequivocally whether or not the use of autologous plasma from human donors who have undergone filtration leukopheresis can be used to increase granulocyte yields if given prior to a subsequent pheresis. It has already been demonstrated by other groups that it is possible to improve granulocyte yields in humans by stimulation of the donor granulocyte count with the use of potent hormones (generally adrenocorticosteroids) or red cell sedimenting agents given before granulopheresis (1-4). In fact, with most systems, it is the only means by which granulocytes in sufficient number to obtain a clinical effect can be harvested. The use of such hormones in normal donors is not without risk. Because of the lack of a safer method for mobilizing granulocytes in donors and because of the serious nature of granulocytopenia in patients with a deficiency in their immune response, this risk has been accepted in the absence of a safer method, and the long-term effects of hydroxyethyl starch are still being questioned. Ideally, a normal physiological mechanism for stimulation of the donor granulocyte count would be preferred.

We have shown previously in rats (5-7) that it is possible to stimulate the donor granulocyte count and to improve granulocyte yields by taking advantage of normal humoral homeostatic mechanisms.

Collection of rat granulocytes by filtration leukopheresis results in such efficient and rapid removal of granulocytes from the circulating pool that granulopoietic mechanisms are stimulated to mobilize granulocytes from body reserve stores (5). We have hypothesized that these cells are released not only from the peripheral marginating pool but also from the bone marrow reserves. Our calculations suggest that on the basis of available evidence the numbers of granulocytes

which can be mobilized are too great to have come only from the marginating pool which is considered to be approximately equal to circulating pool (8).

The studies carried out under the present contract have been designed to elucidate the nature of the mechanisms for mobilizing granulocytes under the impetus of rapid depletion of the circulating pool and to provide some understanding of the kinetics of this mobilization.

Prior to clinical studies to determine the possibility of using the phenomenon of granulocyte mobilization to increase granulocyte yields in human donors, we have been making every effort to determine the kinetics of granulocyte mobilization under these very special conditions. We have determined that within certain circumscribed contexts, the animal model for filtration leukopheresis developed by us is analogous to the human system. Some of the studies carried out during this reporting period further address this question. There are certain logistic problems concerning the optimal use of time and materials for making the best use of this animal model for the kind of definitive investigations we are planning during the next phase of these studies. These investigations will ultimately attempt to suggest the optimal approach to clinical studies designed to provide the greatest granulocyte yields with the system of filtration leukopheresis. In this regard, we have investigated further the effect of the characteristics of the test animal, the rat, on the results obtained. Further, we have tested additional factors such as depth of anesthesia, anticoagulant, and the interaction of the circulating blood and the plastic conduits of the filtration system, per se, on granulocyte mobilization.

We have retested control materials (homologous plasma) and potential control materials (heterologous plasma) so that any contribution they may make to the results can be assessed and taken into account when granulocyte increments are calculated.

We have investigated whether the rat model reacts to a drug in the same way as do human beings as an additional check on the similarity of the rat to the human being in this regard.

A further study of the kinetics of granulocyte mobilization and the degree to which such mobilization occurs during filtration leukopheresis has been carried out using a mathematical model which is descriptive of actual mobilization of cells and takes into account not only the cells in the circulating blood but also those removed during the filtering process. This study provides basic information on granulocyte kinetics.

Finally, in collaboration with Dr. Frederick Rice at American University, Washington, D.C., and with Dr. Harvey Bank at the Medical University of South Carolina, Charleston, South Carolina, attempts are being made to characterize by chemical and physical means the humoral factor(s) in normal plasma and plasma from donors who have undergone filtration leukopheresis.

METHODOLOGY*

Effect of anesthesia, anticoagulant, and blood/plastic interaction on granulocyte mobilization

The animal model upon which all the filtration experiments are based requires that the animals be anesthetized, that they be anticoagulated, and that the blood come in intimate contact during each circuit through the system with plastic cannulae and the plastic walls of the filter which contains the nylon wool (9,10). Heretofore, the effect of the interaction of any or all of these modalities have not been assessed to determine whether they contribute to changes in the granulocyte count by stimulating granulopoietic mechanisms and thereby contribute to the granulocyte increments observed during and following filtration leukopheresis. Alternatively, they have not been assessed for their ability to inhibit such mechanisms.

In order to determine the effect of these modalities on granulocyte mobilization, four groups of animals were studied:

1. Ten rats given only the usual induction anesthesia - 60 mg/kg of pentobarbital sodium (Nembutal) intraperitoneally (i.p.).
2. Ten rats given 60 mg/kg of Nembutal i.p. and 400 I.U. of Heparin sodium intravenously via the exposed femoral vein.
3. Ten rats given 60 mg/kg of Nembutal, i.p., 400 I.U. of Heparin i.v., and having an arteriovenous fistula between the carotid artery and jugular vein identical to that created for filtration of the blood through nylon wool except that the arterial and venous cannulae were connected directly,without the intervening filter (10).

*All the studies described in this report were carried out using normal Sprague-Dawley rats.

4. Fifteen animals treated exactly as those in Group 3 except that the nylon wool filter was a part of the system, i.e., the normal animal model for filtration leukopheresis.

A granulocyte count and differential were obtained as soon as the animals were anesthetized but before anticoagulation or insertion of the cannulae. Since Heparin is given immediately after insertion of the cannulae and just before filtration is begun, the Heparin was given after the animals in Groups 3 and 4 had been cannulated and the flow of blood through the system was begun. Synchronization of all groups was attempted by making certain, within narrow limits, that animals requiring only injections were prepared so that they were anesthetized and heparinized at approximately the same time as those requiring surgery. Since the injections and surgery are quick and simple, there was less than a 10 minute difference between the groups requiring surgery and those requiring only injection in terms of the beginning and ending of the experiments.

Effect of depth of anesthesia on granulocyte increments

Previous studies under this contract (11) have used as controls, rats which have been anesthetized and to which nothing else has been done except tail snips for WBC and differential counts for up to five hours. Routinely, one sees among many of these groups a slow but consistent increase in the granulocyte count over time. Consideration has been given to the possibility that this increment in the count results in some way from repetitive tail snips. However, it has also been considered that in spite of the appearance of constant, deep anesthesia, slow but imperceptible recovery from deep anesthesia may be triggering hormonal activity, especially adrenal (cortical or medullar), which could account for the observed granulocyte increment. Apart from basic information regarding kinetics of granulocyte mobilization, the answer to this question could suggest that the number

of samples ought to be reduced. If, on the other hand, recovery from deep anesthesia caused a granulocyte increase, repetitive increments of anesthetic may be indicated or the duration of some experiments may be shortened.

In the present experiments, two groups of animals were studied:

1. Thirty-seven animals were given only induction anesthesia - 60 mg/kg Nembutal, i.p.
2. Twenty-one animals were given induction anesthesia at the same dose but an additional bolus of 6 mg/rat (0.1 ml) was given intraperitoneally at one, two, three, and four hours after induction.

Following induction of anesthesia, WBC and differential counts were obtained immediately after and every 30 minutes following induction of anesthesia. The changes in granulocyte count were calculated from these measurements.

Effect of anesthetic and normal homologous plasma on the granulocyte count

We have shown that a small but consistent increment in the granulocyte count (<1,000/mm^3 during a five-hour period) occurs during prolonged deep anesthesia induced by repetitive boluses of anesthesia. We have also shown on many occasions (11) that injection of normal rat plasma as control for studies involving treatment with postpheresis plasma (PPP) was accompanied by a small but discrete increment in the granulocyte count in animals given only induction anesthesia. In light of the results of the studies described above, it was felt that we should determine whether the granulocyte increments previously obtained in animals given normal homologous rat plasma under only induction anesthesia is caused by the plasma or whether this increment was, in fact, a result of lightening of the anesthesia over the period

of the experiment. To answer this question, two groups of animals were studied:

1. Five normal rats were given only induction anesthesia plus an additional bolus of 6 mg/rat of Nembutal i.p., one, two, three, and four hours after induction of anesthesia.
2. Ten normal rats were anesthetized in the same way as the Group 1 animals. In addition, each of these rats was given 1.5 ml/kg of normal homologous plasma immediately after anesthesia was induced.

A WBC count and differential were obtained immediately after induction of anesthesia and each 30 minutes for the subsequent five hours. The granulocyte count was calculated from these measurements for each time period.

Effect of human plasma on granulocyte counts in the rat

The use of normal plasma as control for studies involving effects of modified rat plasma (PPP) is time consuming and expensive to prepare. Normal human plasma can be obtained cheaply in large quantities, and the plasma from a single human donor could serve as control for many of the experiments using the rat model. To determine whether heterologous (human) plasma could be used as a control for experiments involving the use of modified rat plasma or whether it could be used in place of rat plasma for elution of rat granulocytes from nylon filters, the following experiments were done. Three groups of rats were studied:

1. Nine animals were given 0.5 ml/kg of normal human plasma intravenously.
2. Nine animals were given 1.0 ml/kg of normal human plasma intravenously.

3. Nine animals were given 1.5 ml/kg of normal human plasma intravenously.

WBC counts and differentials were obtained before and each 30 minutes for five hours following plasma injection. Granulocyte counts were calculated from these measurements.

Effect of intravenous injection of prednisolone on the granulocyte count of normal rats

Prednisolone injection is often used in human donors to mobilize granulocytes in the circulating granulocyte pool to improve granulocyte yields (12). To determine whether the rat model for filtration leukopheresis is equivalent to the human being in this regard, human prednisolone (an adrenocorticosteroid) was administered intravenously to normal rats. This hormone is known to be effective in demarginating granulocytes in the human being within a few hours. The object of this experiment was to determine: (1) whether the rat responds in the same way to human prednisolone; and (2) if so, how rapidly and to what degree.

Three normal rats were anesthetized in the usual manner. A WBC count and differential were obtained. A dose of 20 mg/kg of prednisolone was injected via the exposed femoral vein. WBC counts and differentials were obtained every 30 minutes for three hours thereafter. The granulocyte counts for each time period were calculated from these measurements.

Effect of animal weight (age?) on the normal granulocyte count

We had shown previously (7) that donor weight (age?) was an important determinant, within limits, of the granulocyte increments seen during filtration leukopheresis of the rat. Animals with low

weights,on the average,mobilized fewer granulocytes during a circumscribed period of time than those with intermediate weights. We had also shown that animals with low prepheresis granulocyte counts produced fewer granulocytes than did animals with higher counts (7).

To correlate these observations, an analysis of the last 150 pheresed animals was carried out to determine whether there was any relationship between animal weight and normal (pretreatment) granulocyte count under the conditions of these experiments. The analysis was made by dividing the rats into four groups:

1. Six rats weighing less than 300 grams.
2. Thirty rats weighing 300 to 399 grams.
3. Thirty-four rats weighing 400 to 499 grams.
4. Eighty rats weighing 500 grams or more.

The mean pretreatment granulocyte count for each of these groups was calculated.

Theoretical versus actual changes in granulocyte count after filtration leukopheresis in the rat

To obtain some insight into the real effect of filtration leukopheresis to mobilize granulocytes in the donor, the study described below was done.

Past experience (10) has demonstrated that a measurable degree of granulocytopenia followed by a mild granulocytosis can be expected within the first 30 minutes of pheresis. It has also been shown (10) that granulocyte removal of blood passing through the filter within the first 30 minutes to be not less than 80%. We had also determined (5) that depending on the size and condition of the animal, blood flow through a standard filter (1 cm I.D., 6 cm in length, filled with 1.47 grams of nylon wool) ranges between 0.5 and 1.5 ml/minute.

Nine normal rats were anesthetized and pheresed as described previously (10). The change in granulocyte count during the first 30 minutes was determined (at 5, 10, 15, 20, 25, and 30 minutes during the pheresis).

Although actual flow rates were not measured in the present experiments, periodic examination was made visually to determine adequacy of flow rate.

A mathematical model was constructed which suggested the changes in granulocyte count which occurred in the absence of mobilization under conditions of flow rates of 1.3, 1.0, and 0.7 ml/minute. The difference between the expected count under circumstances of no mobilization and the actual counts seen in the nine animals suggested a more realistic value for granulocyte mobilization during the first 30 minutes of pheresis than was suggested by the counts in the animals themselves. For example: Thirty minutes of pheresis at 1.3 ml/min. equals 39 ml of blood processed. At an efficiency of 80% granulocyte removal, this would be equivalent to removal of all granulocytes from 31 ml of blood. The blood volume in the size animals being used in these experiments is less than 30 ml. If no granulocytes were mobilized during this procedure, all the granulocytes would be removed from the circulating blood within 30 minutes. This is obviously not the case. In fact, as stated earlier, a leukocytosis occurred before the end of the 30 minute pheresis. This study was intended to further elucidate the kinetics of granulocyte mobilization during the process of filtration leukopheresis in the rat.

Collaborative studies with Dr. Rice on the characterization of the granulopoietic factor in PPP

Dr. Frederick Rice of American University in Washington, D.C., has studied problems of leukocyte mobilization for many years. He has described a material obtained from plasma which he has designated

"leukogenenol" (13). This material, when injected into normal animals, stimulates a generalized leukocytosis which involves both a lymphocytosis and a granulocytosis. At the request of Dr. Robert Jennings of ONR, we provided Dr. Rice with plasma from rats which had been leukopheresed, as well as some from normal nonpheresed animals. Some of the aliquots provided were obtained from animals immediately after termination of pheresis. Other aliquots were obtained from animals 24 hours after termination of pheresis. Dr. Rice assayed these plasmas for the presence of leukogenenol.

WBC and differential counts were obtained from each pheresed animal before pheresis and immediately after pheresis was terminated. The granulocyte increment was determined from these measurements with the objective of determining if leukogenenol was measured whether the titer could be correlated with the degree of granulocytosis.

Collaborative studies with Dr. Bank on the characterization of the granulopoietic factor in normal plasma

Following discussions with Dr. Harvey Bank of the Medical University of South Carolina at the Cryobiology Meeting in Atlanta in October 1979, a collaborative study was instituted to attempt to characterize the granulopoietic factors in normal plasma. Two aliquots of 35 ml of pooled normal rat plasma were prepared and sent to Dr. Bank in dry ice. He performed certain chemical fractionation procedures on one aliquot of the plasma and returned them together with the unfractionated control plasma. A total of 22 fractions was returned. Since this investigation was designed as a blind study, the manipulations used in preparing these fractions are not yet known to us. They are coded. We have just started to examine these fractions to determine their ability to mobilize granulocytes following injection into normal rats.

Four normal rats were anesthetized with Nembutal, 60 mg/kg, i.p. A WBC count and differential were obtained. Each animal was injected with 1.0 ml/kg of Fraction "D" except for animal #4 which received only 0.67 ml/kg because of lack of more material. This fraction was chosen arbitrarily. A WBC count and differential were obtained each hour for five hours after injection. The change in granulocyte count for each time period was calculated from these measurements.

RESULTS

Anesthesia, heparin, blood/plastic interaction, and the granulocyte count

The average increases in granulocyte count of 10 rats which had been given only an induction dose of anesthetic intraperitoneally were 873 and 4,725/mm^3 after one and two hours respectively (Fig. 1). When Heparin anticoagulation was added to anesthesia immediately after induction of anesthesia, the average increases in granulocyte count for 10 animals one and two hours after this procedure were 2,335 and 5,867/mm^3, respectively. The addition of an arteriovenous fistula to the anesthetized, anticoagulated rats by means of a plastic cannula between the carotid artery and jugular vein in 10 animals resulted in mean granulocyte increments one and two hours after initiation of this procedure of 3,585 and 8,692/mm^3.

These changes in the granulocyte count were compared with those observed in 15 animals which were anesthetized, anticoagulated, and in which a two-hour filtration leukopheresis was performed. In this group, the mean granulocyte increment after one and two hours of this procedure were 5,990 and 17,400/mm^3, respectively.

Depth of anesthesia and granulocyte increment

As seen in Fig. 2, continued deep anesthesia maintained by hourly intraperitoneal boluses of Nembutal appeared to prevent a significant increase of the granulocyte count for as long as five hours. At the end of that time, the average mean granulocyte increment in 21 animals was 198/mm^3. During the entire five hours, the average mean increment never reached 1,000/mm^3. In the 37 animals which received only induction anesthesia, there was a small but sustained increase in the

mean granulocyte count starting approximately 90 minutes after anesthesia induction which reached approximately 5,000/mm^3 at 3 1/2 hours. This was essentially maintained for the following 1 1/2 hours, although there was a minor drop-off of the mean granulocyte increment.

Anesthesia and homologous plasma on the granulocyte count

The results demonstrated in Fig. 3 suggest that the use of normal rat plasma as a control for experiments in which postpheresis plasma is used contributes little, if any, to the small but consistently seen granulocyte increment observed following its use in normal anesthetized rats.

In comparing the curves representing mean granulocyte increments observed in groups of animals which had received only an induction dose of anesthetic or anesthetic together with normal homologous rat plasma, there are no significant differences at any points on the curves during the five hours of the experiments.

An analysis of variance was calculated for each time point of this experiment. In no instance was a significant difference seen between the group which received plasma and that which did not.

Human plasma in rats

These experiments demonstrate (Fig. 4) that human plasma in doses greater than 1.0 ml/kg when injected into rats stimulates granulocyte mobilization. There is also a suggestion that even at a dose of 1.0 ml/kg, there is some granulocyte mobilization three or more hours after injection. However, this result is confounded by the knowledge that in previous experiments in which homologous plasma, normal saline, or nothing was given, a granulocyte increment equivalent to that seen following injection of 1.0 ml/kg of human plasma was often observed. The suggestion that as little as 1.0 ml/kg of heterologous plasma does,

in fact, result in mobilization of some granulocytes derives from the fact that a greater increment is seen following a dose of 1.0 ml/kg than with 0.5 ml/kg. The curves shown in Fig. 4 suggest that the mobilization of granulocytes by heterologous (human) plasma is both dose- and time-dependent.

Prednisolone on the granulocyte counts of rats

The granulocyte count of three normal rats was increased significantly within 30 minutes of injection. Fig. 5 demonstrates this effect in the three individual rats in which it was injected. As seen in the figure, there is an initial increase in the granulocyte count of all three animals, within 30 minutes of injection which averaged over 10,000/mm^3, and a plateau for an additional 90 minutes. However, at the end of three hours, the granulocyte count in all three rats demonstrated a further significant increase to 16,227/mm^3.

Normal granulocyte count vs body weight (age?)

As seen in Fig. 6A, animals in the lowest weight group demonstrated a mean granulocyte count which was substantially lower than that of the next largest group. The next largest group showed a mean granulocyte count which was higher still. There was no significant difference between the mean normal granulocyte counts of the two heaviest groups.

A similar result is seen when comparing the mean granulocyte increment following filtration leukopheresis except that the heaviest group demonstrated a lower increment than the next lightest group (Fig. 6B).

Theoretical vs actual granulocyte changes after filtration

The actual mean change in granulocyte counts of nine normal rats which underwent 30 minutes of filtration leukopheresis is shown in Fig. 7. As seen previously, there was a granulocytopenia which reached its nadir 10 minutes after initiation of leukopheresis; the beginning of a recovery in the granulocyte count at 15 minutes, and a granulocytosis within 20 minutes after initiation of leukopheresis.

Plotted on the same figure are the theoretical changes in the granulocyte count which would have occurred in the absence of granulocyte mobilization under the conditions hypothesized for these experiments, namely, (1) blood flows of 1.3, 1.0 and 0.7 ml/minute, (2) 80% efficiency of granulocyte removal,and (3) a blood volume of 8% of the body weight. The calculations from which these plots were derived are shown in Table 1.

We suggest that in the absence of any granulocyte mobilization under these conditions, a flow rate of 1.3 ml/minute would have resulted in complete depletion of the granulocytes from the circulating blood following 26 minutes of filtration. At a flow rate of 1.0 ml/minute, the granulocyte levels would have been reduced by approximately 90% in 30 minutes. If the flow rate was 0.7 ml/minute, a 62% depletion of the granulocyte levels would be expected after 30 minutes of filtration. In fact, the granulocyte count seen after 30 minutes of filtration was at the pretreatment level.

Fig. 8 shows the actual granulocyte counts in these nine animals and suggests the hypothetical counts under the conditions described above.

On the basis of these experiments, it is possible to calculate, at least within an order of magnitude, the number of cells mobilized

during any time period. For example, a rat with a blood volume of 27 ml and a granulocyte count of 1,754 (the mean of the counts after 20 minutes of pheresis in these experiments) would have 4.74×10^7 circulating granulocytes. Theoretically, according to this model, at a circulation rate of 1.3 ml of blood per minute through the filter and an efficiency of granulocyte removal of 80%, the circulating granulocyte count would be approximately 300/ml or a total circulating granulocyte pool of about 0.81×10^7. One could then calculate a mobilization of 3.93×10^7 granulocytes.

Measurement of leukogenenol in PPP

On three occasions, aliquots of postpheresis plasma and normal (control) plasma were sent to Dr. Frederick Rice for measurement of leukogenenol. As seen in Table 2, the leukogenenol level in the plasma drawn from animals immediately after pheresis was equal to that of the controls. However, in those animals pheresed 24 hours previously, there was a significant level of leukogenenol measured. The plasma level from the animal which we calculated to have a moderate level of neutrophil releasing factor on the basis of the granulocyte increment at the end of pheresis showed a leukogenenol level which was twice that of the control (normal) animal. The plasma level from the rat which we calculated to have a high level of NRF demonstrated a leukogenenol level of three times that measured in the control.

Granulocyte mobilizing activity of normal plasma fractions

To the present time only one of the plasma fractions provided by Dr. Harvey Bank has been assayed for granulocyte mobilizing activity. Fig. 9 demonstrates the effect of this plasma fraction on the granulocyte counts of four normal rats during the three hours following injection. There was a slight mean increase in the granulocyte count ($2,000/mm^3$) two hours after injection. This increased to about $9,000/mm^3$ by three hours. It should be noted, however, that results from animal to animal were very inconsistent.

DISCUSSION

These studies and those carried out previously under this contract have been aimed towards developing a useful method for improving granulocyte production by taking advantage of the body's own normal homeostatic mechanisms. For these animal studies to provide meaningful data which can lead to appropriate clinical studies, it is essential that many possible confounding influences on the results of the use of our animal model for filtration leukopheresis be investigated so that they may be anticipated and accounted for when clinical trials begin.

The primary emphasis of these studies since our last Annual Report to ONR on March 31, 1979 has been placed on: (1) defining as much as possible the confounding influences which could confuse the results and lead to erroneous interpretations of the data; (2) further investigations of normal homeostatic mechanisms for granulocyte mobilization during filtration leukopheresis; (3) determining characteristics of the neutrophil releasing factor elaborated during leukopenia secondary to granulocyte removal from the circulating blood; and (4) an additional comparison of the animal model with the human mechanisms for mobilizing granulocytes.

The results described in this report extend and further elaborate on those carried out previously.

We have seen previously that animals to which nothing has been done except induction of anesthesia with Nembutal exhibit a slowly rising granulocyte count with time. We have investigated the reason for these observations.

These studies show that continued deep anesthesia maintains the granulocyte count at near baseline levels in the absence of any granulocyte mobilizing stimulus. It is also clear that a single induction dose of anesthesia begins to "wear off" at around 1 1/2 to 2 hours after induction. All the animals given only the single initial dose of anesthetic appeared to be essentially in deep anesthesia, although occasionally the forelimbs would be seen to twitch. Since the only difference between the two groups of animals was the additional anesthetic in the second group, it must be assumed either that the Nembutal itself has a direct inhibitory effect on granulocyte mobilization or that the animals not given additional anesthetic were slowly awakening, although the awakening was not obvious. We feel that the latter mechanism is the operative factor.

It is reasonable to assume, and consistent with known physiological events, that as the animals start to recover from the anesthetic (get "lighter") there is increased adrenocortical activity. Corticosteroids are known to mobilize granulocytes from the marginating to the circulating pool (5).

These studies are important in suggesting at least two approaches in carrying out and analyzing the kinds of experiments being done within the context of this contract.

1. Maintain a basal level of granulocytes by maintaining deep anesthesia so that any increase in granulocytes by granulocyte mobilizing agents may not be confounded by normal physiological mechanisms, or
2. Accept a level of defined increment as the base level at various times after induction of anesthesia and make the calcualtions of increments on this basis.

We had heretofore questioned the etiology of this consistently seen increase in the granulocyte count among control animals. We had suggested that Heparin in the infused control plasma could account for this increment. Heparin was not given to the animals in the present study.

Since a variety of manipulations of the animals are intrinsic to the carrying out of filtration leukopheresis studies, we determined the contribution of the additive effect of these manipulations in normal animals. We have shown that lightening of anesthesia alone can result in mobilizing granulocytes in the circulating pool. Additional studies described herein have also demonstrated that Heparin given to anesthetized animals has no significant granulocyte mobilizing effect. However, when an artificial fistula is created by directing blood flow through plastic cannulae from the carotid artery to the jugular vein, there is a significant increase in the granulocyte count within two hours in otherwise normal animals which have been anesthetized and anticoagulated with Heparin. This does not, however, approach the granulocyte increment observed when a nylon filter is added to the system. It is possible that the interaction of the blood with the nylon fibers in the filter cause the very substantial further increment in granulocyte count between the sham-pheresed and pheresed animals (8,000 vs 17,000/mm^3), although this is unlikely. Within the context of these experiments even such an event would not invalidate the elaboration of a presumably endogenous granulocyte mobilizing factor which can be transfused with the plasma into donor animals and which is ultimately capable of stimulating a granulocytosis in those animals. We have shown, then, that many alterations in the normal physiology of rats may result in the mobilization of some granulocytes. However, the most potent impetus for increased mobilization of granulocytes among all the perturbations studied is removal of granulocytes from the circulation (induction of a granulocytopenia). This study also suggests that

very careful analysis of "background" granulocyte mobilization, having nothing to do with the body's response to granulopheresis, must be undertaken when experiments of this nature are carried out.

Normal homologous plasma is often used as a control for studies in which postpheresis plasma is the test material. It was essential to determine whether the slight but consistent elevation of the granulocyte count seen following its injection is a reflection of an intrinsic effect of the plasma or, as suggested in experiments described above, is a result of lightening of the anesthesia. These studies suggest that normal homologous plasma has no effect on the granulocyte count for at least five hours after injection and can, therefore, be used as control for PPP injection without concern for granulocyte mobilizing activity.

On the other hand, heterologous plasma is not an appropriate substitute for homologous plasma in this kind of experiment. In the volumes which would normally be used in these experiments (1.5 ml/kg), a substantial granulocyte mobilizing effect was observed following its injection. It was not possible in these studies to determine the mechanism of its effect although one could suppose that it is the direct result of the introduction of foreign proteins into the animals.

The observation that intravenous injection of prednisolone, a glucocorticoid, is capable of exerting a granulocyte mobilizing effect is not remarkable. It does show that the animal model is equivalent to the human being in that this hormone is capable of exerting a granulocyte mobilizing effect. What is remarkable, however, is the rapidity with which it exerts its activity in the rat. Pretreatment of human granulocyte donors with prednisolone and other similar steroids has generally been shown to require several hours to exert a significant effect on the granulocyte count of normal human beings. Although

its greatest effect was seen three hours after injection into rats, a significant increase in the granulocyte count ($\sim$8,000/mm^3) was seen within 30 minutes after injection. Whether the rat is particularly sensitive to corticosteroids in this regard or whether, again, it is in part an effect of the introduction of a foreign protein could not be determined by these experiments.

The demonstration that small animals on the average have low normal granulocyte counts is important. Since we have shown that granulopheresis of animals with low granulocyte counts results in a lower yield of granulocytes (5), this correlation demonstrates that studies of this sort can be altered to a significant degree by selecting animals of varying weight and granulocyte counts. It is essential that such factors be considered when carrying out and analyzing the data derived from such studies. Again, because of the large differences between control animal granulocyte counts over time and those from animals undergoing granulopheresis or those treated with postpheresis plasma, no essential changes in our conclusions from earlier studies need be made.

A simple enumeration of granulocyte counts and measurement of changes in the granulocyte counts of individuals during granulopheresis gives no indication of the degree of mobilization of granulocytes from body stores. If, during the course of granulopheresis, there was absolutely no change in the granulocyte count over a period of time, it would appear that no activity was taking place among the various granulocyte compartments. It would be obvious, however, that if cells are being removed from the circulating blood at a rapid rate, mobilization of cells from other compartments would be required just to maintain a level granulocyte count. Further calculations are necessary to elucidate the degree of such movement among the compartments. A mathematical model has been constructed to suggest the magnitude of granulocyte mobilization during filtration leukopheresis.

This model can be used to calculate the numbers of mobilized granulocytes in single animals, although it was used in these experiments to suggest orders of magnitude of mobilized cells among a group of rats. Such a model can be invaluable in studies of granulocyte (or other cellular) kinetics.

Dr. Frederick Rice has been attempting to characterize granulopoietic substances in the blood. He has extracted a material from plasma which he has called "leukogenenol." He considered that the granulocyte mobilizing factor which we have described as being elaborated during filtration leukopheresis may be leukogenenol. The results of his measurements of plasma from leukopheresed rats suggests that this is not the case. Considerable granulocyte mobilizing activity is consistently seen when plasma from rats which have been leukopheresed for as little as one hour is injected into normal rats. Dr. Rice was not able to detect increased levels of leukogenenol in plasma from rats pheresed for two hours. The fact that this material was identified in increased amounts in plasma obtained from rats 24 hours after termination of leukopheresis suggests that this is a different factor which may be concerned with longer term granulocyte homeostasis. It is not unreasonable to assume that leukogenenol may work at a lower level of granulopoiesis, i.e., at the maturation level of the cascade.

Further studies will be required to elucidate these questions and further collaborative efforts with Dr. Rice appear to be justified. Such a collaborative effort may be fruitful in shedding new light on a slowly emerging area of blood cell physiology.

The studies being carried out in collaboration with Dr. Bank have not yet reached a stage where conclusions regarding them can be drawn. In the interest of good science, it was agreed that this would be a blind study. We have received more than a score of plasma fractions

from Dr. Bank. One of these had been tested for granulocyte mobilizing activity several weeks ago. The results of the use of that fraction are equivocal (Fig. 9). A systematic study of the remaining fractions has just been started. We expect to complete this study during the next several weeks.

CONCLUSIONS AND SIGNIFICANT ACCOMPLISHMENTS

Several manipulations of the animals are necessary to carry out the procedure of filtration leukopheresis or to study the effect of postleukopheresis plasma in normal animals. They are all anesthetized. Pheresed animals are always anticoagulated with Heparin. The plasma injected into PPP recipients contains Heparin. Blood is in intimate contact with plastic tubing for extended periods during the filtering process. Recovery from anesthesia can result in granulocyte mobilization. It is not clear from these experiments whether or not Heparin mobilizes granulocytes. However, it would appear that if it does, its contribution to increases in the granulocyte count is small. Of more consequence is the interaction of blood (probably the cellular elements) with the plastic tubing. However, the contributions of all these factors together is less than half of that of granulocyte removal as a cause of granulocyte mobilization. These factors do not in any way minimize the importance of the previously reported observations regarding granulocyte mobilization in leukophered animals and those given PPP. However, their potential contribution to granulocyte changes in these systems must be taken into account when interpreting the results of such experiments. One of these mechanisms can be minimized or eliminated as a factor in these studies if desired, i.e., maintenance of deep anesthesia can eliminate lightening of the anesthesia and subsequent stimulation of granulocyte mobilization by this means.

The injection of normal homologous plasma does not mobilize granulocytes but heterologous plasma does. The use of human plasma for any purpose in these studies is contra-indicated. The glucocorticoid, prednisolone, exerts a granulocyte mobilizing effect in the rat but at a much more rapid rate than is generally seen in the human being.

Smaller (younger?) rats have a lower basal granulocyte level than older rats. This suggests that for the purposes of these studies, larger (older?) rats should be used to obtain the optimal effect whenever the objective is to obtain the greatest number of granulocytes since it has been shown that the higher the prepheresis count, the greater the granulocyte yield. The construction of a mathematical model for measuring the actual degree of granulocyte mobilization will allow more precise measurements of movement of granulocytes within the granulocyte compartments of the body. The "neutrophil releasing factor" described by us as the operating hormonal mechanism for rapid mobilization of granulocytes in animals rendered leukopenic by filtration leukopheresis is probably not "leukogenenol." It would appear that this latter substance is elaborated much later than "neutrophil releasing factor." We expect to be able to determine shortly which fraction(s) of normal plasma is capable of stimulating release of granulocytes from the marginating pool (if, in fact, normal plasma contains such a factor). If such a factor is not present in normal plasma, this will be of major physiological significance.

The following papers have been published, presented, or accepted for publication:

1. Roy, A. Granulopoietic activity and physiologic properties of postleukapheresis plasma. Presented at the Symposium on Storage and Preservation of Granulocytes, 16th Annual Meeting, Society for Cryobiology, Atlanta, Georgia, October 1979.

2. Roy, A. Kinetics of neutrophil releasing activity of filtration leukapheresis and postleukapheresis plasma. Cryobiology, in press.

3. Lionetti, F., Hunt, S., Schepis, J., Roy, A., Liss, R., and Valeri, C. In vivo distribution of cyrogenically preserved guinea pig granulocytes, Cryobiology, in press.

REFERENCES

1. Higby, D., Mishler, J., Rhomberg, W., Nicora, R., and Holland, J. The effect of a single or double dose of dexamethasone on granulocyte collection with continuous flow centrifuge. Vox Sang. 28: 243, 1975.

2. Glasser, L. Functional considerations of granulocyte concentrates used for clinical transfusions. Transfusion 19:1, 1979.

3. Isbister, J., Scurr, R., Clouten, V., and Biggs, J. Use of Dextran 150 as a macromolecular agent to improve granulocyte yields on the intermittent flow blood cell separator (Haemonetics Model 30). Transfusion 19:583, 1979.

4. Strauss, R., Goedken, M., Maquire, L., Koepke, J., and Thomson, J. Combined platelet-leukapheresis:A technique for preparing separate platelet and granulocyte-platelet units from single donors. Transfusion 19:206, 1979.

5. Roy, A., Brivkalns, A., and Yankee, R. Use of postpheresis plasma to improve granulocyte yields for transfusion. Blood 45:345, 1975.

6. Roy, A. and Ramirez, M. Increased granulocyte counts in recipients of plasma from leukopheresed rats: A dose response study. Exp. Hemat. 6:375, 1978.

7. Roy, A. Annual report to ONR. March 31, 1979.

8. Boggs, D. Transfusion of neutrophils as prevention or treatment of infection in patients with neutropenia. N. Engl. J. Med. 290: 1055, 1974.

9. Roy, A., Djerassi, I., Franklin, A., and Brivkalns, A. Animal model for the study of filtration leukopheresis. Fed. Proc. 32: 871, 1973.

10. Djerassi, I., Roy, A., Franklin, A., and Brivkalns, A. Filtration leukopheresis in the rat. Exp. Hemat. 2:336, 1974.

11. Roy, A. Unpublished observations.

12. Pflieger, H., Dietrich, M., Arnold, R., and Wildfleur, A. High granulocyte yield with a simple method of filtration leukapheresis. Klin. Wschr. 56:409, 1978.

13. Rice, F. Personal communication.

TABLE 1

THEORETICAL GRANULOCYTE REMOVAL FROM RATS DURING FILTRATION LEUKOPHERESIS*

Duration of Pheresis (minutes)	Blood Flow Per Minute (ml)	Total Volume of Blood Pheresed (ml)	Efficiency of Removal (%)	Volume of Blood with Complete Gran. Removal (ml)	Granulocytes Removed (%)
5	1.3	6.5	80	5.2	19
10	1.3	13.0	80	10.4	39
15	1.3	19.5	80	15.6	58
20	1.3	26.0	80	20.8	77
25	1.3	32.5	80	26.0	96
30	1.3	39.0	80	31.2	116
5	1.0	5.0	80	4.0	15
10	1.0	10.0	80	8.0	30
15	1.0	15.0	80	12.0	44
20	1.0	20.0	80	16.0	59
25	1.0	25.0	80	20.0	74
30	1.0	30.0	80	24.0	89
5	0.7	3.5	80	2.8	10
10	0.7	7.0	80	5.6	21
15	0.7	10.5	80	8.4	31
20	0.7	14.0	80	11.2	41
25	0.7	17.5	80	14.4	53
30	0.7	21.0	80	16.8	62

*Based on a blood volume of 27 ml.

TABLE 2

LEUKOGENENOL LEVELS IN POSTPHERESIS AND NORMAL PLASMA

Animal No.	Probable N.R.F.* Titre	Leukogenenol Measurement (μg/l)
1	High	7
2	Low	8
3	None (normal)	7
4	Low to moderate	6.5
5	None (normal)	8
6	Moderate	11.3
7	High	15.3
8	None (normal)	5.3

Animals 1, 2, and 4 - Plasma obtained immediately after termination of pheresis.

Animals 6 and 7 - Plasma obtained 24 hours after termination of pheresis.

*Neutrophil releasing factor.

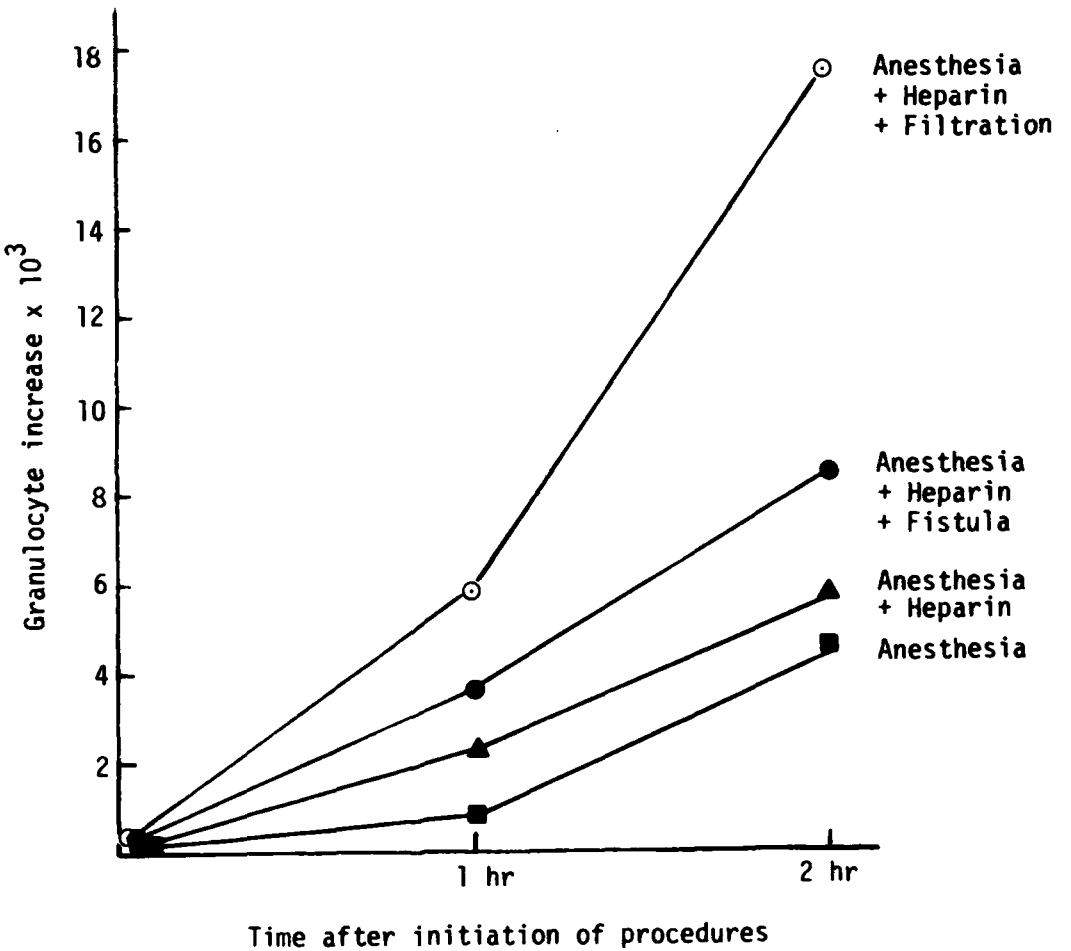

Fig. 1. Effect of various procedures on the granulocyte count of normal rats

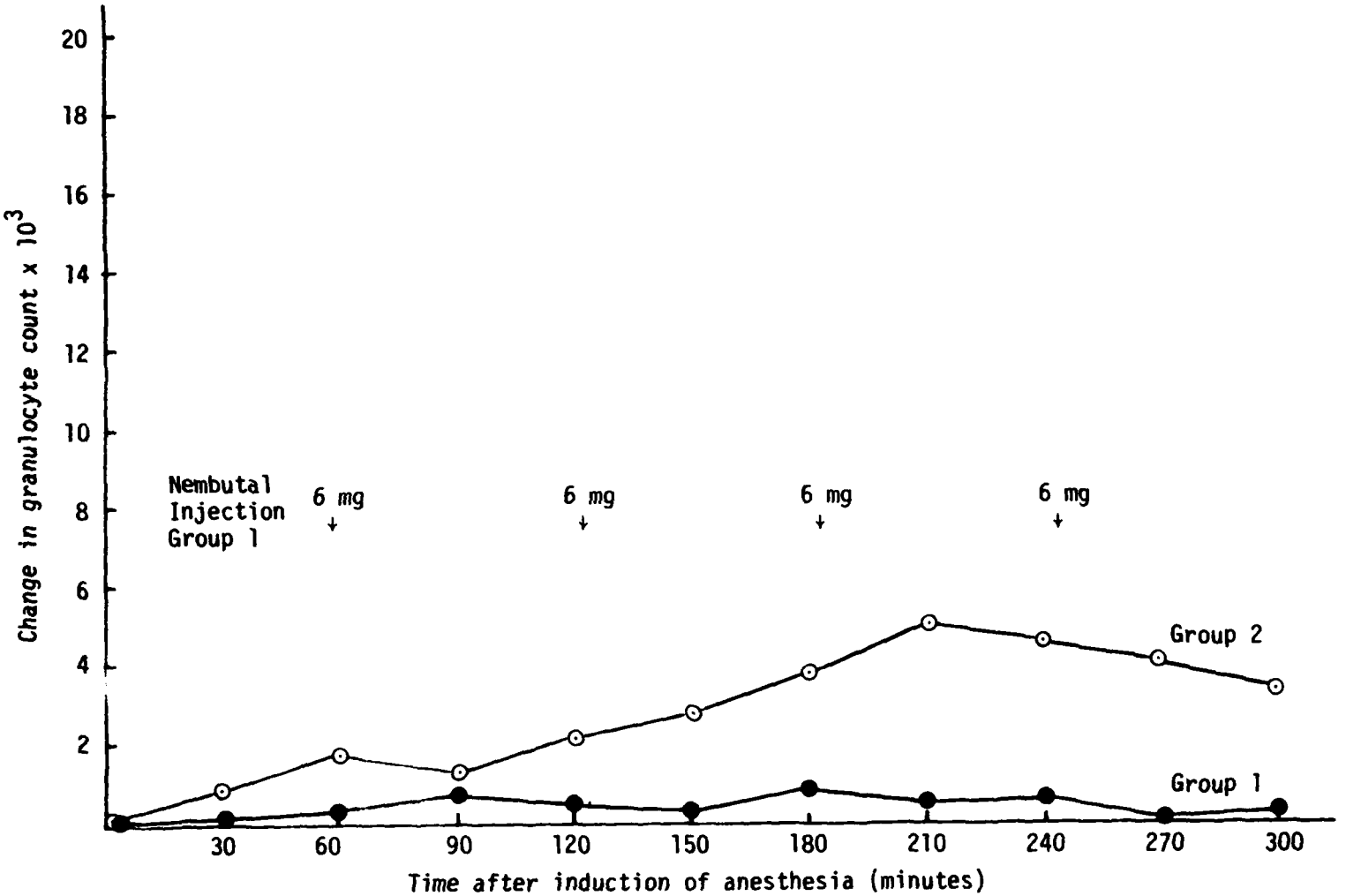

Fig. 2. Effect of maintenance of deep anesthesia on the granulocyte counts of normal rats

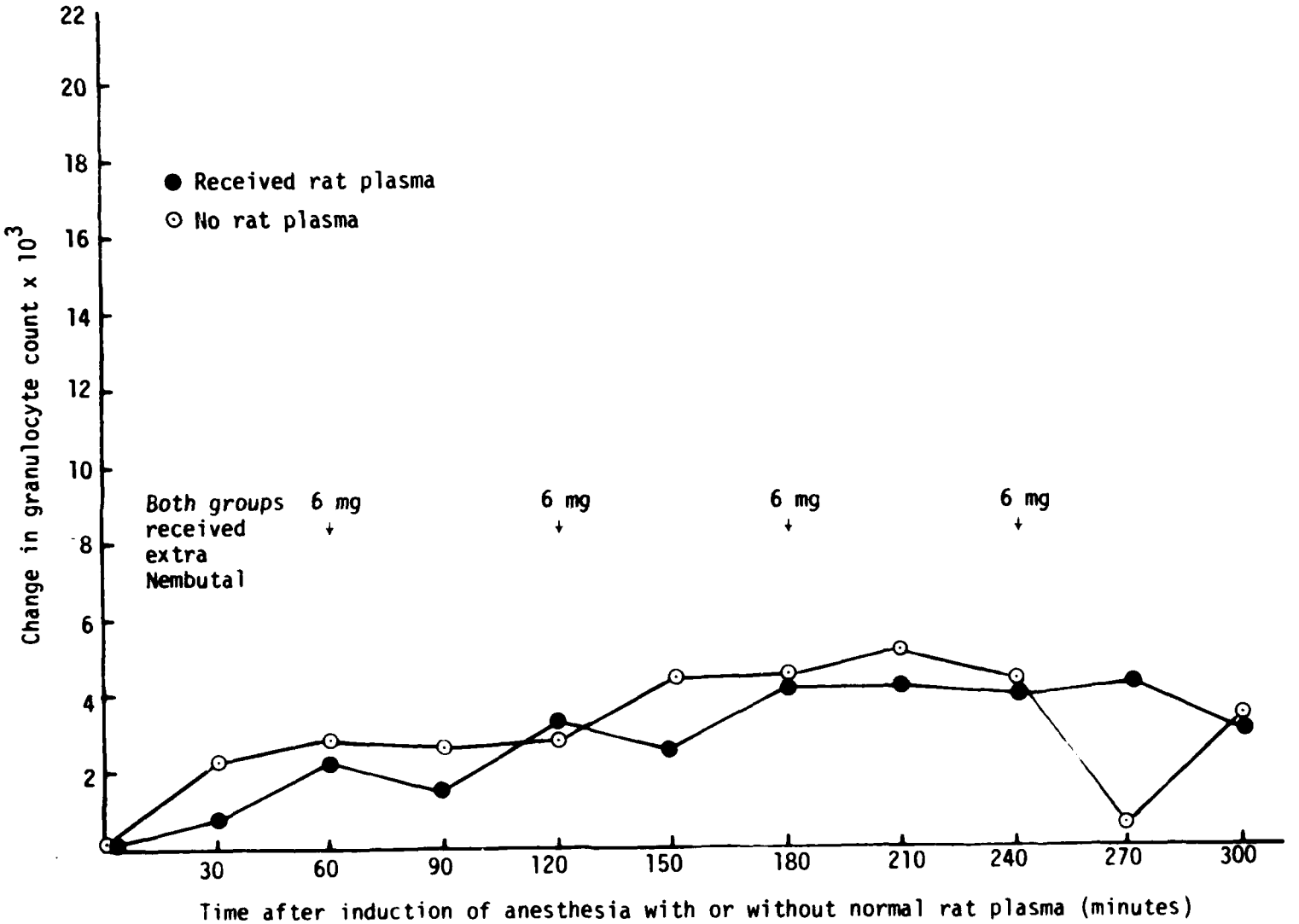

Fig. 3. Effect of fresh homologous rat plasma on the granulocyte counts of normal rats given repeated injections of Nembutal

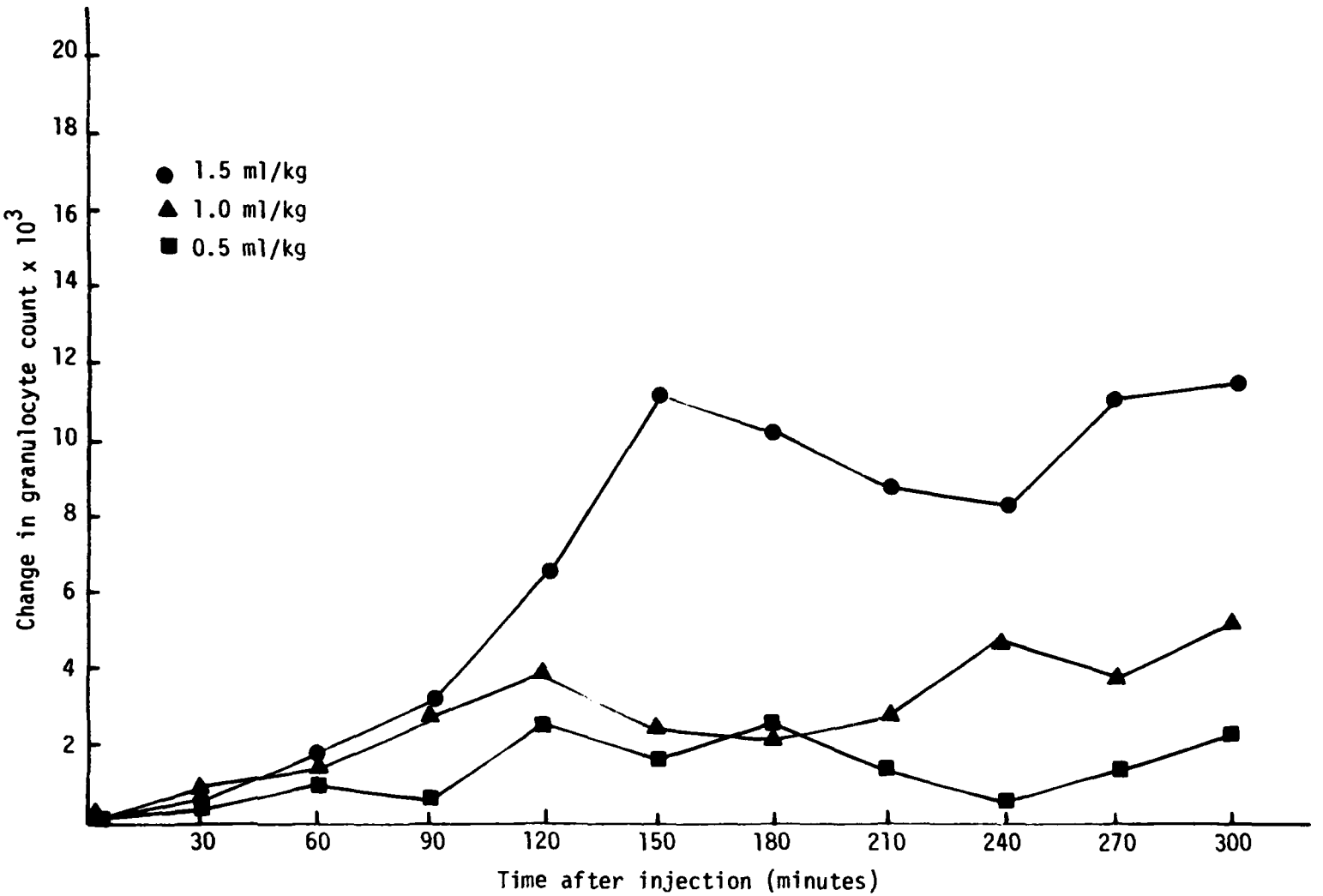

Fig. 4. Effect of normal human plasma on the granulocyte counts of normal rats

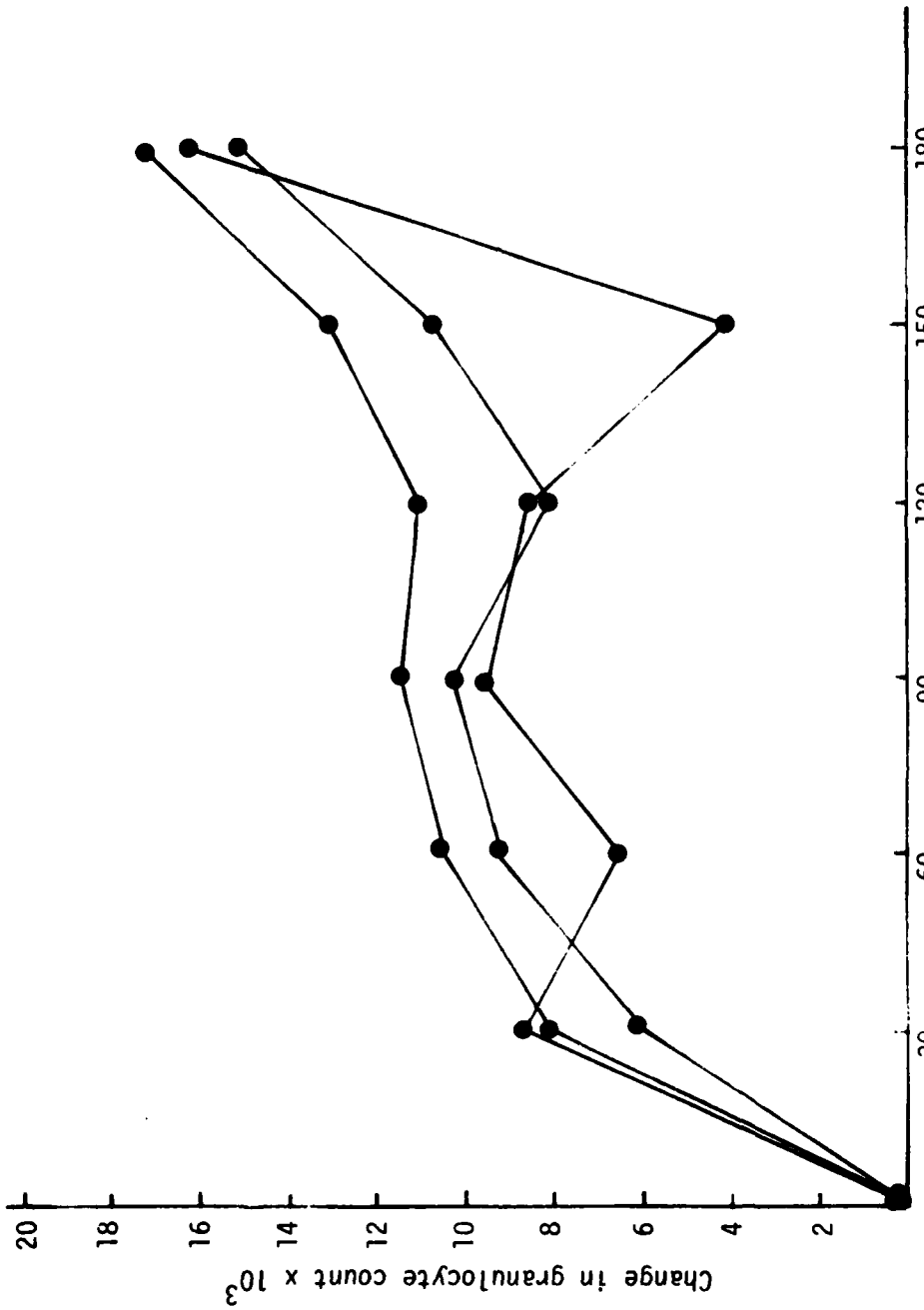

Fig. 5. Effect of prednisolone on the granulocyte counts of normal rats

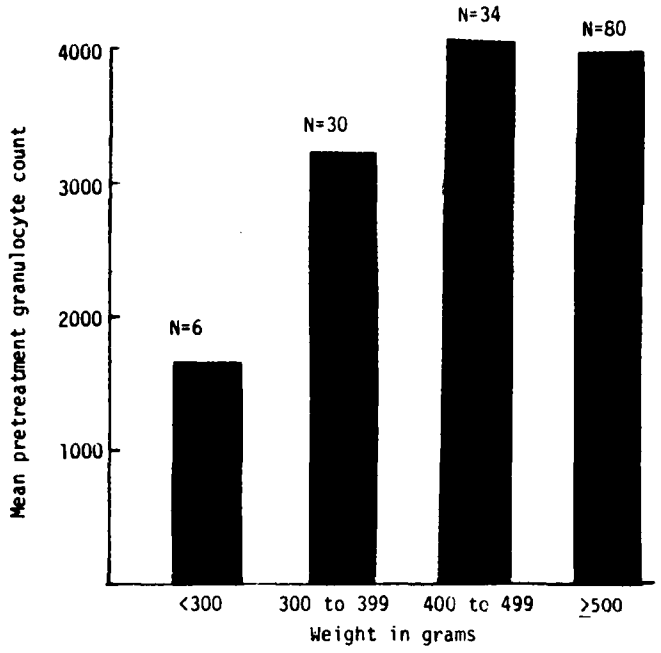

Fig. 6A. Pretreatment granulocyte count vs donor weight

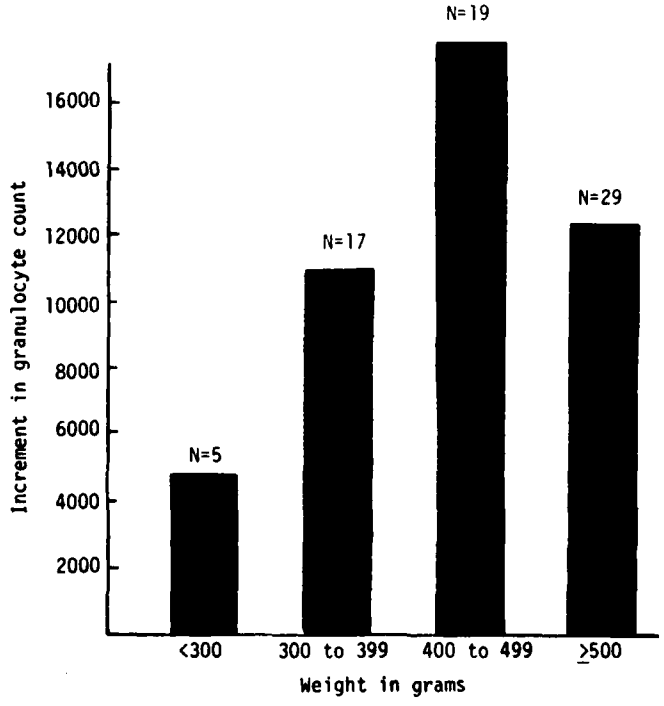

Fig. 6B. Increment of granulocyte count in leukopheresed rats vs donor weight

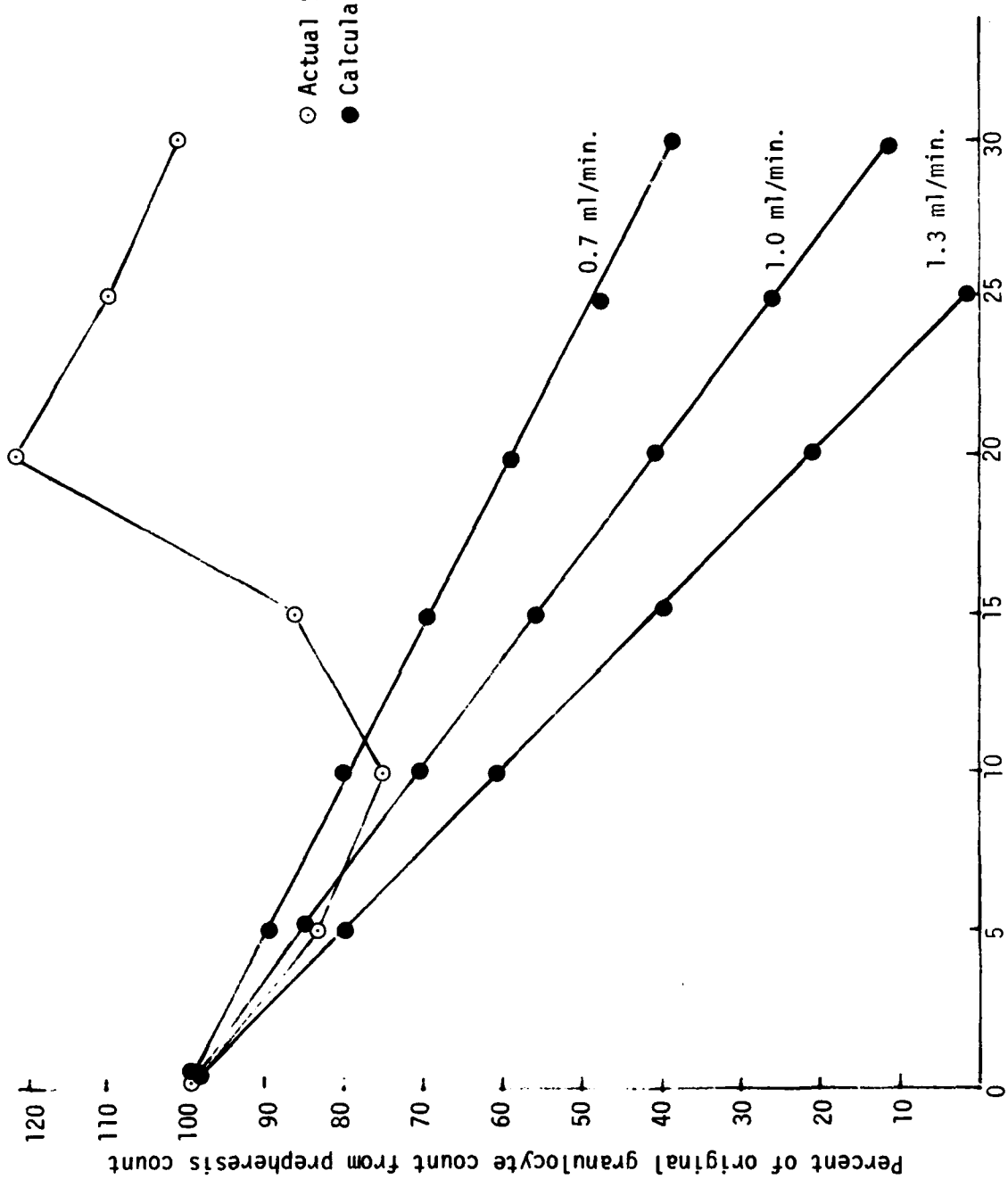

Fig. 7. Actual and hypothetical granulocyte changes in rats during filtration leukopheresis

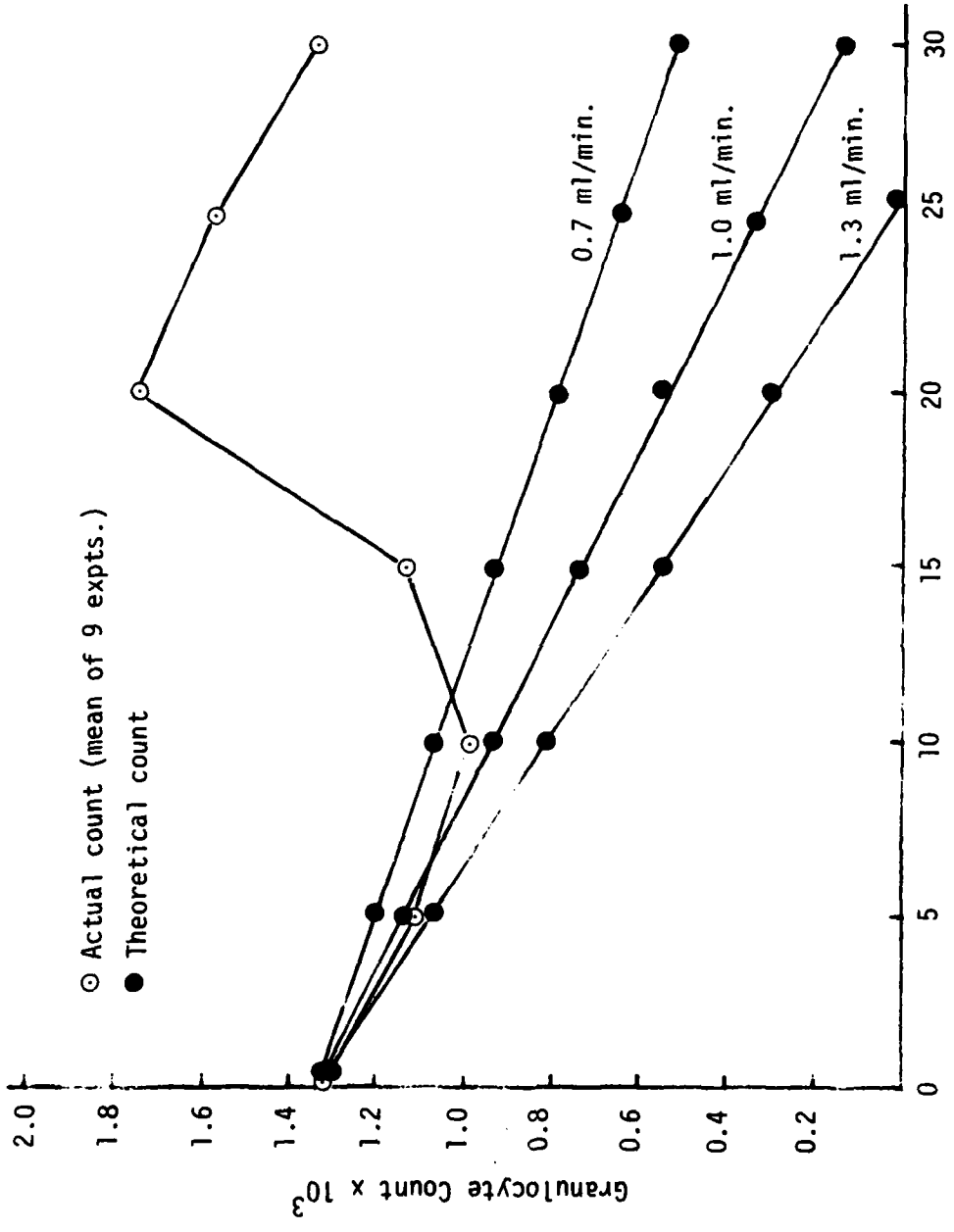

Fig. 8. Actual and theoretical counts of rats at different rates during filtration leukopheresis

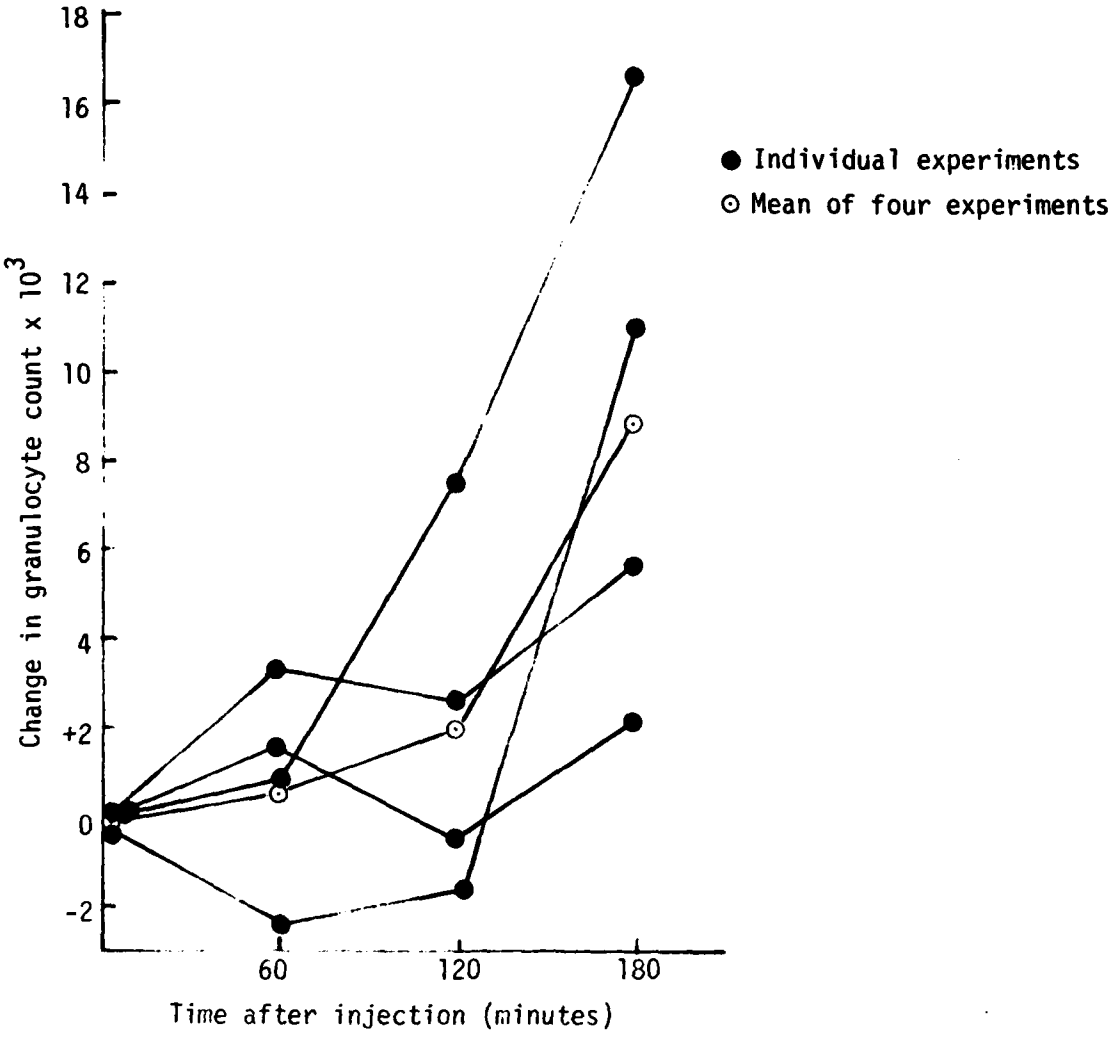

Fig. 9. Effect of plasma fraction D on the granulocyte count of normal rats

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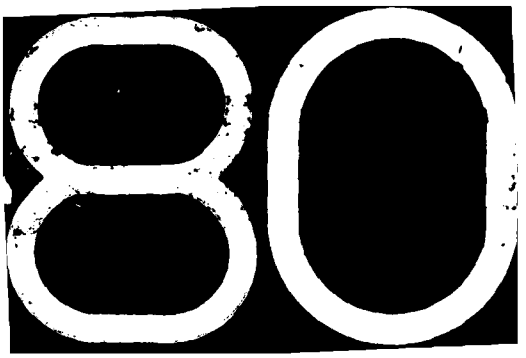
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